

# Influence of the genus *Bacillus* bacteria on the content of H<sub>2</sub>O<sub>2</sub> and the activity of hydrolases and their inhibitors in potato plants during *Phytophthora infestans* Mont. de Bary infection

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**Abstract.** The authors studied the effect of treatment with bacteria *Bacillus subtilis* Cohn (strains 26D) and *B. thuringiensis* Berliner (strain B-6066) on the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content, the activity of hydrolytic enzymes and their protein inhibitors in potato plants (*Solanum tuberosum* L.) in connection with development of resistance to the late blight pathogen – oomycete *Phytophthora infestans* Mont. de Bary. Studies were carried out on potato plants of the susceptible Early Rose potato cultivar that were treated with a suspension of *B. subtilis* and *B. thuringiensis* bacteria (10<sup>8</sup> cells/ml) and infected with *P. infestans* (10<sup>7</sup> spores/ml). A decrease in the degree of leaf damage by oomycete was revealed under the influence of the genus *Bacillus* bacteria, depending on the strain. The increase in potato resistance to *P. infestans* infection was mediated by the stimulating effect of the *B. subtilis* 26D and the *B. thuringiensis* B-6066 bacteria on the concentration of H<sub>2</sub>O<sub>2</sub>, the modulating effect on the activity of hydrolytic enzymes and the enhancement of the transcriptional activity of protease and amylase inhibitor genes in plant tissues. Differences in the degree of activation of the transcriptional activity of hydrolase inhibitor genes by the *B. subtilis* 26D and the *B. thuringiensis* B-6066 bacteria were revealed, which suggests differential ways of forming the potato resistance to *P. infestans* under their influence.

## 1 Introduction

One of the ways of increase of plant resistance to pathogenic organisms and adverse environmental factors by ecologically friendly methods is the scientifically substantiated use of non-pathogenic rhizospheric bacteria (PGRP – plant growth promoting rhizobacteria). In this regard, especially attractive are the bacteria of the genus *Bacillus*, that are highly

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effective, undemanding to cultivation media, retaining their viability for a long time, and able to activate natural defense mechanisms in a plant organism [1-2].

A lot of compounds have been developed based on *Bacillus* bacteria in order to protect plants from phytopathogens and pests, the effect of which is based on the natural antagonism of *Bacillus* to many phytophagous organisms [3]. Thus, the active basis of biological products against a wide range of food crop pests is a complex of protein crystals of delta-endotoxin and thermostable exotoxin of the microbial culture *Bacillus thuringiensis* var. *thuringiensis*. The “Fitosporin” compound was created based on the cell culture, metabolites and bacterial spores of *Bacillus subtilis* 26D, which is used to protect plants from pathogens with various types of parasitism. An important feature of biological products based on living microorganisms is the non-specific activation of plant defense mechanisms [4].

At present, a large amount of literature on development decrease of phytopathogens under the influence of *Bacillus* bacteria is presented, however, the mechanisms of the induction of plants’ protective reactions when treated with these bacteria still remain unclear. The ability of some representatives of the genus *Bacillus* to multiply inside plant tissues is known [5], as well as their ability to secrete hydrolytic enzymes that inhibit the pathogens development [6]. Among the metabolites of *Bacillus* bacteria, cyclic low molecular weight lipopeptides, such as surfactin and iturin [7] were distinguished, inducing the generation of hydrogen peroxide and an oxylipin signaling system in plants that regulates the activity of proteinase inhibitors [8].

The objective of this work is a comparative study of influence of the *Bacillus subtilis* Cohn and *B. thuringiensis* Berliner bacteria on the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content, the activity of hydrolytic enzymes and the transcriptional activity of the genes of hydrolase inhibitors in potato plants during infection with late blight pathogen *Phytophthora infestans* Mont. de Bary.

## 2 Materials and methods

### 2.1 Objects of study

We used potato plants grown from micro tubers of the susceptible Early Rose variety. The tubers were planted in containers with soil (TerraVita, peat of varying degrees of decomposition, purified river sand, perlite, complex mineral fertilizer, biohumus; pH 6.0-6.5). Plants were grown on a light site for 15 days, then inoculated with a suspension culture (final titer of 10<sup>8</sup> cells/ml) of *B. thuringiensis* B-6066 bacteria, obtained from the State Research Institute of Genetics and Selection of Industrial Microorganisms, and *B. subtilis* 26D bacteria from the commercial biological product “Fitosporin-M” (“Bashinkom”, Russia). 5 days after bacteria treatment, the plants were infected with a spore suspension (10<sup>7</sup> spores/ml) of *P. infestans* – the late blight pathogen. *P. infestans* (Bashkiria strain) was grown in test tubes on potato-glucose medium for 11 days. In order to obtain zoospores from zoosporangia, the fungus mycelium was poured with distilled water and kept for 30 min at 4 °C, and then 20 min at room temperature. We used the culture of the oomycete *Phytophthora infestans* (Mont.) de Bary from the collection of the Institute of Biochemistry and Genetics of the Ufa Federal Research Centre of the Russian Academy of Sciences. After 24, 48, and 72 h after infection, the control and infected plants were fixed for the biochemical studies. Bacteria were cultured for 24 hours in Luria-Bertani liquid medium, then the suspension was diluted with distilled water to the required concentration. The development of the disease was observed for 7 days and was estimated by the % ratio of the damage area to the leaf blade area. The leaves were photographed, images were analyzed in the ImageJ program (NIH, USA).

## 2.2 Determination of H<sub>2</sub>O<sub>2</sub> content

The leaves were homogenized in 0.025 M phosphate buffer, pH 6.2 (PB), in a 1:3 ratio, centrifuged for 20 min at 10,000 g in a centrifuge (“Eppendorf”, Germany). The supernatant was used to determine the H<sub>2</sub>O<sub>2</sub> content. The H<sub>2</sub>O<sub>2</sub> content was evaluated at 560 nm by using xylenol orange. The reagent contained 0.074% Mohr's salt in 5.81% sulfuric acid and 0.009% xylenol orange in 1.82% sorbitol (in a ratio of 1:100). The optical density of the reaction products was measured on a Biospek-Mini spectrophotometer (“Shimadzu”, Japan).

## 2.3 Determination of enzymes and inhibitors activity

The activity of proteases, amylases, and inhibitors was determined by hydrolysis of a substrate immobilized in a polyacrylamide gel [9].

## 2.4 Evaluation of the transcriptional activity of potato protease and amylase inhibitor genes

RNA from plants was isolated by using trizol (Molecular Research Center, Inc., USA). In order to obtain cDNA based on the mRNA of the studied samples, a reverse transcription reaction was performed using M-MuLV reverse transcriptase according to the protocol of the supplier. The accumulation of transcripts of the amylase inhibitor genes (GenBank number XM006351484) and the proteinase inhibitor (GenBank number JX683427) was analyzed by real-time quantitative PCR using the iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, USA) and intercalating dye SYBR Green I (“Synthol”, Russia). Changes in transcriptional activity of genes (estimation of the number of mRNA copies for each gene) were determined by the St\_act relative reference gene (“housekeeping gene”, actin, GenBank number X55749) using the iCycler iQ5 Real-Time Detection System software (“Bio-Rad”, USA). A computer analysis of amino acid and nucleotide sequences was performed using the Lasergene computer software package from DNASTAR, Inc (USA).

## 2.5 Statistical data processing

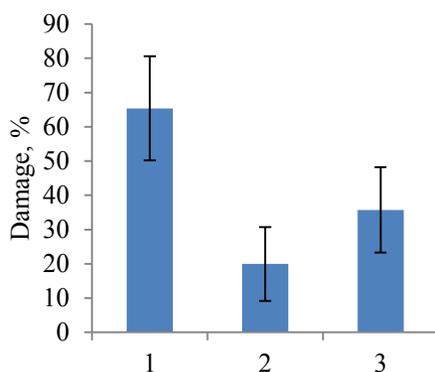
The experiments included at least three biological repeats in analysis of the biochemical parameters and at least 15 repeats in analysis of the transcriptional activity. Figures show sample averages and their 95% confidence intervals.

# 3 Results and discussion

## 3.1 Influence of genus *Bacillus* bacteria on the *P. infestans* damage of potato leaves and their H<sub>2</sub>O<sub>2</sub> content during *P. infestans* infection

7 days after inoculation with *P. infestans*, the damage degree of plant leaves in the control variant was 65.1±6.3% (Fig. 1). In the experimental variants with bacteria treatment, the severity of symptoms of late blight was reduced: down to 20.3±2.7% when treated with the *B. subtilis* 26D strain and down to 35.8±3.8% when treated with the *B. thuringiensis* B-6066 strain. As it can be seen from Fig. 1, the studied strains of *Bacillus* bacteria had an inhibitory effect on the growth and development of *P. infestans* in potato leaves. Moreover, the treatment with the *B. subtilis* 26D strain was 15% more effective than the treatment with the *B. thuringiensis* B-6066 strain. Thus, a decrease in the damage degree of potato plants by *P. infestans* oomycete was revealed under the influence of *Bacillus* bacteria, depending on the

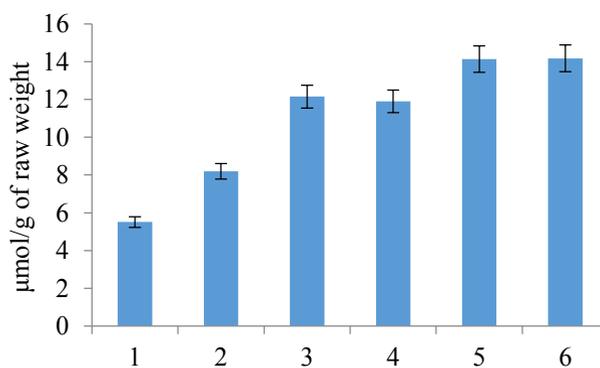
strain. It is known that endophytic bacteria are capable of triggering a chain of defense reactions in plants by synthesizing various metabolites with hormonal and signaling properties [7-8].



**Fig. 1.** Influence of *Bacillus* bacteria on the *P. infestans* damage of potato leaves. 1 – control variant, 2 – *B. subtilis* 26D treatment, 3 – *B. thuringiensis* B-6066 treatment.

One of the mechanisms for increasing potato resistance to late blight pathogen *P. infestans* is associated with a hypersensitivity reaction, which suggests the generation of ROS in plants in response to the pathogen introduction. One of the earliest reactions of plant cells to contact with a pathogen is the production of ROS, which are involved in intracellular signaling through the interconversion.

Studies have shown that inoculation of *P. infestans*, treatment with the *B. subtilis* 26D and the *B. thuringiensis* B-6066 increased the level of H<sub>2</sub>O<sub>2</sub> in potato plants (Fig. 2). Moreover, treatment with the *B. thuringiensis* B-6066 bacteria had a more significant stimulating effect on H<sub>2</sub>O<sub>2</sub> production in healthy plants, and with the *B. subtilis* 26D – in the infected plants.

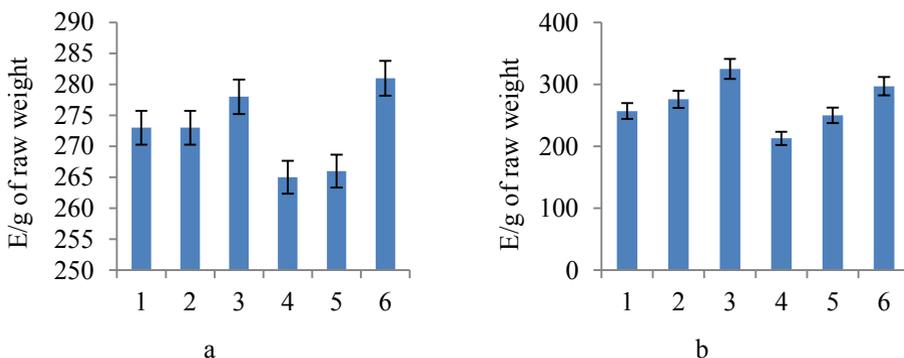


**Fig. 2.** H<sub>2</sub>O<sub>2</sub> content in potato leaves when treated with *Bacillus* bacteria and infected with *P. infestans*, 48 hours after infection. 1 – control variant, 2 – *B. subtilis* 26D treatment, 3 – *B. thuringiensis* B-6066.

Hydrogen peroxide is a secondary messenger involved in the work of various plant cell signaling systems and regulating the expression of protective protein genes [10]. By stimulating the accumulation of ROS, endophytic bacteria are likely to induce the transmission of signals forming a defense mechanisms complex. The participation of bacteria *Bacillus spp.* in the generation of H<sub>2</sub>O<sub>2</sub> and increasing the resistance of wheat to pathogens of common bunt [11] and *Septoria tritici* blotch were shown [12].

### 3.2 Influence of genus *Bacillus* bacteria on activity of hydrolytic enzymes and their inhibitors in potato plants during *P. infestans* infection

Hydrolytic enzymes of plant pathogens play an important role in their introduction and distribution in the plant organism [13]. Exogenous proteases of microorganisms are involved in the destruction of structural proteins of the cell wall [14] and other plant protective peptides [15], as well as in the processing of their own extracellular proteins of microorganisms necessary for the pathogenesis process [16]. An important role of proteases is indicated by the direct dependence of the intensity of the disease in plants on the activity of microbial proteases observed in a number of cases. The level of proteolytic activity is also associated with necrotic processes. The biological databases contain information on the possibility of *P. infestans* expression of many proteases of different families: cysteine and serine proteases, proteases of the Hsl, Fts, and Clp families ([www.ncbi.nlm.nih.gov/bioproject/PRJNA17665](http://www.ncbi.nlm.nih.gov/bioproject/PRJNA17665)).



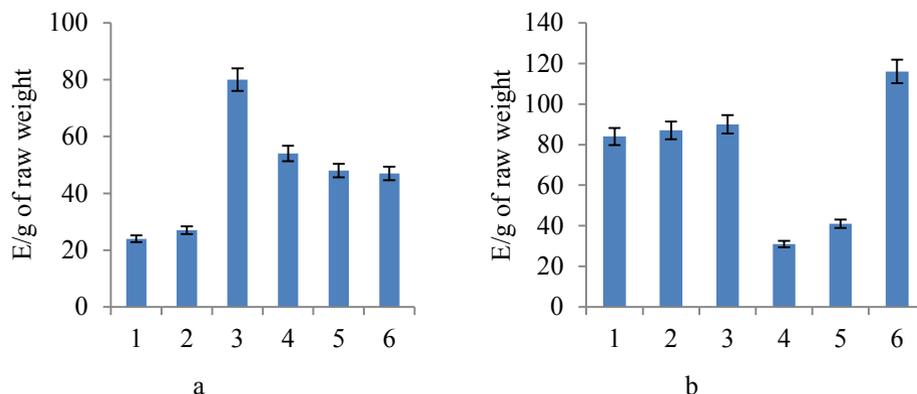
**Fig. 3.** Activity of proteases (a) and amylases (b) in leaves when treated with *Bacillus* bacteria and infected with *P. infestans*, 72 hours after infection. 1 – control variant, 2 – *B. subtilis* 26D treatment, 3 – *B. thuringiensis* B-6066 treatment, 4 – *P. infestans* infection, 5 – *P. infestans* + *B. subtilis* 26D, 6 – *P. infestans* + *B. thuringiensis* B-6066.

As the research results showed, infection of plants with *P. infestans* led to a decrease in the activity of proteases in the potato leaves (Fig. 3a), which could be associated with the activation of their inhibitors (Fig. 5). In healthy and infected potato plants treated with the *B. subtilis* 26D bacteria, protease activity remained at the level of the corresponding control variant (Fig. 3). When plants were treated with the *B. thuringiensis* bacteria, there was a slight increase in the activity of proteases in the leaves. The *B. subtilis* 26D and the *B. thuringiensis* B-6066 bacteria had a similar effect on the activity of amylases in potato plants (Fig. 3b).

Hydrolytic enzymes that cause destruction of the components of the fungal cell wall are widely represented in bacteria of the genus *Bacillus* [17]. To date, the presence of various forms of alpha-amylases with molecular masses of 47 kDa (amyA, amyE genes), 60 kDa (amyS gene) and 75 kDa (amyM gene) has been described in the *Bacillus* bacteria [18-24]. It can be assumed that phytopathogenic fungi and bacteria release a mixture of hydrolases into the environment, the role of which is both in the destruction of plant structures in order to obtain nutrients, and in active interaction with their protective systems.

Hydrolase inhibitors play an important role in protecting plants against the penetration and spread of phytopathogens [25-27]. It is interesting to note that hydrolase inhibitors prevent activity of both microbial enzymes and their own plant enzymes, thereby reducing the level of tissue degradation [28-29].

Infection of potato plants with *P. infestans* and treatment with the *B. subtilis* and *B. thuringiensis* bacteria increased the activity of protease inhibitors in plant tissues (Fig. 4a). For example, activity of protease inhibitors increased 4 times in plants treated with the *B. thuringiensis* bacteria compared to the control variant (Fig. 4a).



**Fig. 4.** Activity of proteases (a) and amylases (b) inhibitors in the potato leaves of Early Rose cultivar when treated with *Bacillus* bacteria and infected with *P. infestans*, 72 hours after infection. 1 – control variant, 2 – *B. subtilis* 26D treatment, 3 – *B. thuringiensis* B-6066 treatment, 4 – *P. infestans* infection, 5 – *P. infestans* + *B. subtilis* 26D, 6 – *P. infestans* + *B. thuringiensis* B-6066.

Plant protease inhibitors are active against phytopathogenic microorganism enzymes. Thus, protein trypsin inhibitors from potato tubers and legumes can inhibit the exogenous proteinases of the phytopathogenic fungus *Rhizoctonia solani* Kuhn [30]. High proteolytic activity not only ensures growth and development of the pathogen by amino acids, but also neutralizes the protective proteins of potato – inhibitors of hydrolases and lectins.

A relatively high level of activity of amylase inhibitors was revealed in healthy potato plants, which remained almost unchanged under the influence of treatment with the *B. subtilis* and *B. thuringiensis* bacteria (Fig. 4b). *P. infestans* infection of plants led to a 4-fold decrease in the activity of amylase inhibitors compared to control variant plants. Level of inhibitor activity increased in potato plants that were treated with bacteria, especially under the influence of the *B. thuringiensis* (Fig. 4b).

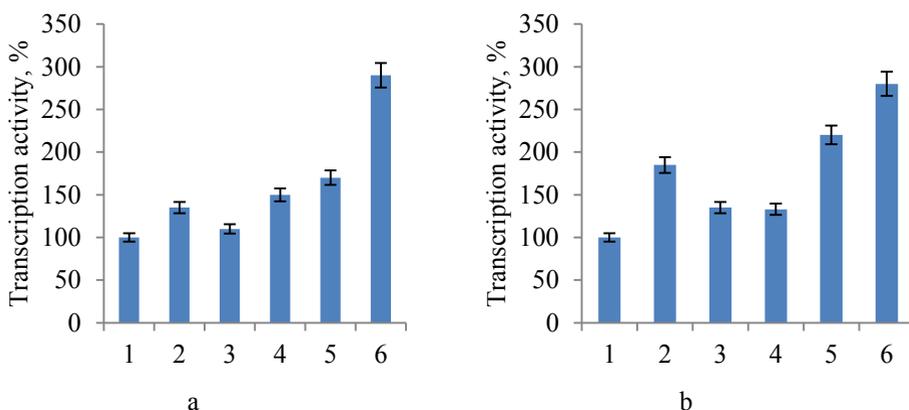
Amylases have been shown to be represented by constitutive proteins in most taxonomic groups of fungi [28]. However, in *P. infestans* amylase is absent. *Phytophthora* phytopathogens activate the biosynthesis of potato's own amylases in the damaged tissues for breaking down starch [31]. It can be assumed that the decrease in the activity of amylase inhibitors in plants is due to the participation of *P. infestans* in the inactivation of amylases, which inhibits the growth and development of the pathogen.

### 3.3 Influence of genus *Bacillus* bacteria on the transcriptional activity of amylase and protease inhibitor genes in potato plants during *P. infestans* infection

The formation of hydrolase inhibitors in response to pathogen incorporation usually occurs de novo. In this regard, we studied the activity of transcription of genes of protease and amylase inhibitors in potato plants infected with *P. infestans* and treated with bacteria of the genus *Bacillus*.

In plants infected with *P. infestans* and treated with *Bacillus* bacteria, accumulation of transcripts of the protease inhibitor gene is observed (Fig. 5a). In plant tissues treated with

the *B. thuringiensis* B-6066, the content of transcripts of the protease inhibitor gene is 3 times higher than that of control variant plants.



**Fig. 5.** Change in the transcriptional activity of the protease (a) and amylase (b) inhibitor genes in potato plants under the influence of *Bacillus* bacteria treatment and infection with *P. infestans*, % ratio related to the control variant (100%), 24 hours after inoculation. 1 – control variant, 2 – *B. subtilis* 26D treatment, 3 – *B. thuringiensis* B-6066 treatment, 4 – *P. infestans* infection, 5 – *P. infestans* + *B. subtilis* 26D, 6 – *P. infestans* + *B. thuringiensis* B-6066.

Similar results were obtained when studying the transcriptional activity of the amylase inhibitor gene during treatment with bacteria and *P. infestans* infection (Fig. 5b). Thus, under the influence of the *B. subtilis* 26D treatment, the level of transcriptional activity in infected plants increased 2.5 times as compared to the control variant, while the *B. thuringiensis* B-6066 treated plants showed an increase of 3 times (Fig. 5b). An increase in the transcriptional activity of the protease and amylase inhibitor genes under the influence of *B. subtilis* and *B. thuringiensis*, as in uninfected plants, but especially during infection, indicates the sensitivity of these genes to the metabolites of *Bacillus spp.* bacteria.

It is known that one of the protective mechanisms of plant cells in response to the introduction of pathogenic organisms is the suppression of the activity of secreted exogenous hydrolytic enzymes by specific plant inhibitors. The widespread occurrence of hydrolase inhibitors in plants of various genera and families confirms their important role in ensuring the vital activity of plants. Hydrolase inhibitors play a significant role not only in increasing the resistance of plants to the effects of phytophagous organisms, but also provide regulation of enzyme activity during growth and development of the plant itself. The possibility of inducing the activity of hydrolase inhibitors in plants upon infection by pathogenic organisms seems to be one of the possible mechanisms for controlling their resistance, including by treatment with compounds based on various strains of the genus *Bacillus* bacteria.

## 4 Conclusion

Hence, the obtained research results indicate that the activation mechanism of protective systems in potato plants by endophytic bacteria of the genus *Bacillus* is mediated by the accumulation of  $H_2O_2$  and increased activity of PR proteins. The revealed differences in the activation of transcriptional activity of protease and amylase inhibitor genes under the influence of the *B. subtilis* 26D and the *B. thuringiensis* B-6066 bacteria suggest differential ways of resistance formation in potato plants to *P. infestans* with their participation.

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